

Predictive Growth of Listeria monocytogenes and  
Aerobic Spoilage Organisms in Raw Chicken  
Packaged in Modified Atmospheres and Air

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## ABSTRACT

The development of Listeria monocytogenes and total aerobic plate counts (APC) on minced raw chicken were compared at 4, 10, and 27°C when packaged in flexible film under both aerobic and anaerobic modified atmospheres (MA) or air. An anaerobic MA (75:25, CO<sub>2</sub>:N<sub>2</sub>) resulted in the failure of both the APC and L. monocytogenes to grow at all temperatures. Both the L. monocytogenes and APC grew to high numbers in air at all temperatures. An aerobic MA (72.5% CO<sub>2</sub>, 22.5% N<sub>2</sub>, 5% O<sub>2</sub>), which more closely duplicates commercial practice, inhibited the increase in APC by more than 4 LOG units compared to air at 4°C. However, the L. monocytogenes was not affected by this atmosphere and increased in numbers by nearly 6 LOG units at 4°C in 21 days. Regression analysis of the LOG growth and 95% confidence intervals showed that the differences between APC and L. monocytogenes in MA were large. The ability of L. monocytogenes to grow in the aerobic MA was not affected by level of the L. monocytogenes inoculum nor by the initial level of APC. These data show that MA packaging of raw chicken (and probably other meats) can substantially inhibit spoilage organisms while allowing pathogenic L. monocytogenes to proliferate.

## INTRODUCTION

Modified and controlled atmosphere packaging (C/MAP) are becoming an increasingly common approach to extend the shelf life of perishable refrigerated foods (NFPA, 1988). C/MAP has become widely practiced in the poultry industry for both raw and cooked products. Several mixed and homogeneous atmospheres have been proposed for C/MAP but the most widely used atmospheres usually contain significantly higher concentrations of carbon dioxide (CO<sub>2</sub>) with or without oxygen (O<sub>2</sub>) and/or nitrogen (N<sub>2</sub>) (Genigeorgis, 1985). Elevated CO<sub>2</sub> concentrations used in C/MAP selectively inhibit the growth of gram-negative spoilage bacteria such as Pseudomonas, Acinetobacter, and Moraxella while allowing many gram positive organisms to proliferate (Silliker and Wolfe, 1980). As a result, the characteristic off-odors, off-colors, and spoilage associated with rapid growth of gram-negative psychrotrophs may be inhibited and product deterioration is slowed. The objective of C/MAP is a 50 to 400% extension of refrigerated shelf life.

The effects of C/MAP on the microbiological safety of foods has not been extensively studied. In the few published reports, several different experimental approaches have been used (Hotchkiss, 1988). Direct inoculation studies as well as more complex studies which attempt to correlate pathogenicity with organoleptic spoilage have been undertaken. Few studies, with the exception of those published by Genigeorgis and coworkers have been designed to be predictive. These workers determined the probability of toxin formation in MAP fish products (Lindroth and Genigeorgis,

1986; Garcia and Genigeorgis, 1987). While their regression analysis provides statistical estimates of the likelihood of toxin formation under different storage conditions, they do not consider the role of spoilage in the safety of C/MAP products.

L. monocytogenes has been isolated from various foods for human consumption including uncooked meat (Kwantes and Isaac, 1971), milk, soft cheese, cabbage, (Schlech et al., 1983), fresh lettuce, celery and tomatoes (Ho et al., 1986). In 1971, Kwantes and Isaac isolated L. monocytogenes from 57% of fresh and frozen poultry samples. Gitter (1976) reported that approximately 15% of the oven-ready poultry samples contained L. monocytogenes. Both fresh and processed meats, including poultry products, have been reported to be commonly contaminated with L. monocytogenes (Brackett, 1988). L. monocytogenes is of interest in refrigerated, extended shelf life foods, such as those packaged in C/MAP, due to its ability to survive and proliferate under adverse conditions, even at refrigeration temperatures, and its ability to grow in microaerophilic environments (Kahn et al., 1975).

The relationship between the growth of spoilage organisms and pathogens is a critical safety factor in C/MAP (Hintlian and Hotchkiss, 1987). The objective of our work was to determine if C/MAP would inhibit spoilage while allowing the growth of L. monocytogenes in raw chicken.

## MATERIALS AND METHODS

### Packaging Materials

Pouches (19 x 14 cm) were constructed from moderate barrier copolymer film (CVP Systems, Inc., Carol Stream, Il.; 0.8 mil Nylon, 1.2 mil ethyl vinyl acetate, and 1.2 mil Surlyn; gas transmission rates [ $\text{cc}/\text{m}^2 \times 24 \text{ h}$ ] were 28 to 38 for oxygen, 4 to 7 for nitrogen and 108 to 128 for carbon dioxide). The volume of the pouch was 880 ml. Two holes were made in the pouch surface and fitted with rubber serum bottle stoppers (Fisher Scientific); the perimeter of the septa was caulked with silicone sealant.

After the pouches were sealed, 2 hypodermic needles (18 gauge) were inserted into one of the septa as a gas outlet. Another needle, connected to the Scott Flowmeter (2-31B Series Gas Blender), was inserted into the second septum. After the pouch was flushed for 60 sec with the desired gas mixture, the inlet and outlet needles were removed. The gas blending system was calibrated by gas chromatography against commercial certified gas mixtures. The minimum flush time required to achieve the desired atmosphere was also determined by gas chromatography. The pouch head space was monitored by gas chromatography on randomly selected pouches throughout the experiment.

### Sample Preparation

Freshly-killed chickens were obtained from the Cornell University Poultry Farm. While on ice, the skin was removed and all meat (white and dark) removed from the bones. The meat was minced for 20 seconds (until homogeneous) in a sanitized Cuisinart. Minced chicken was sealed in Cryovac pouches and frozen until use.

### Standard Curve and Inoculations

L. monocytogenes strain Scott A, serotype 4 (clinical isolate obtained from Dr. Catherine Donnelly, University of Vermont) was maintained on Trypticase Soy Agar with 0.6% Yeast Extract (TSA-YE). Cultures were transferred every fourteen days and incubated at 37°C for 24 hours.

L. monocytogenes was incubated in Trypticase Soy Broth with Yeast Extract (TSB-YE) at 37°C in a shaker bath for 2 hours. Periodically, a small sample of the solution was removed and centrifuged to concentrate the cells. The pellet was resuspended in Phosphate Buffered Saline (PBS). The optical density (o.d.) of the diluted sample was read at 640 nm, using uninoculated PBS as the blank. The culture was further diluted with PBS and plated on Modified McBride Agar (MMA) to determine the number of cells per ml. A standard curve was constructed and the following regression equation ( $R = .93$ ) was calculated:  $\text{o.d.} = 2.9434 + 3.0880 \times (\text{LOG CFU/ml})$ .

### Plating methods for enumeration

The total aerobic plate count (APC) was obtained by pour plate using Plate Count Agar (PCA) (Difco). The plates were incubated at 37°C for 48 hours and colonies counted (Busta et al., 1984).

L. monocytogenes was enumerated on MMA prepared according to the FDA method (Lovett, 1988). The media consists of 35.5 grams of phenylethanol agar (Difco), 10 grams of glycine anhydride (Sigma), 0.5 grams of lithium chloride, and 200 milligrams of cycloheximide (Sigma) per liter of media. The cycloheximide was filter

sterilized and then added to the tempered agar just before pouring plates. The plates were incubated at 37°C for 48 hours. Selected colonies of L. monocytogenes were identified and biochemically confirmed using the FDA procedure (Lovett, 1988). Plating of uninoculated samples did not result in any colony formation.

#### Experimental protocol

One colony of L. monocytogenes Scott A was incubated in 15 ml of TSB-YE overnight at 37°C. The solution was centrifuged, the pellet resuspended in 7 ml of PBS, and the o.d. was measured. The solution was diluted with PBS until the o.d. was 1.0. The concentration of L. monocytogenes with an o.d. equal to 1.0 was determined by standard curve. The solution was further diluted to cell densities of  $10^4$  and  $10^2$  CFU/ml. Ten  $\mu$ l of each inoculum was directly plated on MMA to verify the accuracy of the dilutions; 10  $\mu$ l of PBS was plated as a control.

Duplicate one gram (+/- 0.005 g) samples of raw minced chicken were placed in the wells of sterile deep-well tissue culture plates (Falcon 3046). The surface of the chicken was inoculated with 10  $\mu$ l of one of the cell suspensions (or PBS for APC determination) using a sterile micropipet. Water was added to a separate well to prevent drying. The six-well uncovered tray was placed in a pouch and the sides of the pouch were heat-sealed. The pouch was flushed with one of the atmospheres to be tested or air and incubated at 4, 10, or 27°C. At sampling time, the entire chicken sample from each well was aseptically transferred and serially diluted in PBS and plated on MMA (inoculated) or PCA (uninoculated). Selected



colonies of L. monocytogenes were identified and biochemically confirmed using the FDA procedure (Lovett, 1988). All plating was done in duplicate and each experiment was repeated twice.

Recovery of L. monocytogenes from the chicken was no different from direct plating of the broth on MMA in two trials (two replicates each). The precision of L. monocytogenes recovery from chicken (4 trials, two replicates each) was  $3.23 \times 10^5$  (s.d. = 0.054) CFU/g.

#### Statistical analysis

A statistical analysis was performed over the linear portion of the log growth phase of the curves in order to estimate growth curves for L. monocytogenes and the APC organisms under each set of environmental conditions. The curves were then used to determine the differential growth of the organisms under each set of experimental conditions. Separate linear regression equations were computed for L. monocytogenes and APC organisms for each set of conditions using the SAS package (SAS, 1985) and 95% confidence intervals (CI) were used to assess the accuracy of the prediction equations.

### RESULTS AND DISCUSSION

Both APC and L. monocytogenes failed to grow at an inoculation level of  $10^2$  CFU/g in an anaerobic atmosphere containing 75:25, CO<sub>2</sub>:N<sub>2</sub> at 4, 10, and 27°C, respectively (Table 1). L. monocytogenes is a facultative anaerobe (Holt, 1984), however, we failed to

detect growth on chicken in the presence of the high CO<sub>2</sub> concentration. In air, both L. monocytogenes and APC organisms grew to high numbers (Table 1). At 27°C, there was immediate logarithmic growth, whereas at 10 and 4°C, there were increasing lag phases, respectively. L. monocytogenes proliferated at all temperatures in the presence of air.

There was a rapid increase in the APC in air. However, the elevated aerobic CO<sub>2</sub> atmosphere (72.5% CO<sub>2</sub>, 22.5% N<sub>2</sub>, 5% O<sub>2</sub>) had a strong inhibitory effect on the APC. This effect was much more pronounced at the lower temperature (Table 2). For example, at 4°C, the LOG APC/g in air increased from 4.63 to 9.27 in 21 days, while in the microaerophilic atmosphere, the LOG APC/g only increased from 4.42 to 5.16 in 21 days. The LOG CFU/g for L. monocytogenes increased from 2.57 to 8.5 in both atmospheres during 21 days at 4°C.

Plots of the mean counts showed that the growth curves for each type of organism were non-linear (not shown). At lower storage temperatures, the curves showed typical sigmoidal growth curves. However, at higher temperatures, logarithmic growth was immediate and the samples deteriorated too rapidly for observation to be possible in the lag growth phase (Tables 1 and 2). For this reason, and because a large number of sampling times are needed to determine the parameters of a sigmoidal curve, linear regression was used to fit the linear portion of the log growth phase. While this analysis undercounts organisms during the initial lag phase, and overestimates in the asymptotic phase, it is a good fit to the

growth curve in the area of most rapid growth (Figures 1 and 2).

The regression equations and 95% confidence intervals of the data in Table 2 for the growth in air and MA (72.5% CO<sub>2</sub>, 22.5% N<sub>2</sub>, 5% O<sub>2</sub>) of APC (Figure 1) and L. monocytogenes (Figure 2) were compared at 4°C. The growth of APC organisms was severely inhibited by the MA compared to air. After only 5 days of storage, the 95% CIs diverged. Severe spoilage of raw chicken takes place at an APC of approximately 10<sup>8</sup> CFU/g (Baker et al., 1986). The regression equations (Figure 1) indicated that in air the chicken would reach this point between day 13 and 14 which is what would be expected in commercial practice. However, extrapolation of the growth rate curves showed that the APC in the MA would not reach 10<sup>8</sup> CFU/g until 66 days. This substantial increase in shelf life for MAP poultry has been previously reported (Baker et al., 1986).

In strong contrast to the APC regression equations, the regression equations for the growth of L. monocytogenes in the aerobic MA and air were not different (Figure 2). The ability of L. monocytogenes to grow at reduced O<sub>2</sub> tensions and refrigeration temperatures in media and some foods is well documented (Brackett, 1988; Doyle, 1988). What is important about the present data is the comparison of APC and L. monocytogenes in the two tested MAs. These data illustrate a case where the development of the normal spoilage microflora is strongly inhibited while a potentially fatal food-borne pathogen develops rapidly under what is would be considered excellent refrigeration temperature.

The data (Table 2) from the same experiments conducted at 10 and

27°C were similar with two exceptions. First, both the APC and L. monocytogenes CFU increased at a much faster rate. At 27°C, in air, the APC reached  $10^8$  CFU/g in <8 hr and the L. monocytogenes CFU/g reached  $10^4$  in <7 hr. The second difference was that the MA had a much smaller effect on the APC. The temperature dependence of MA on the inhibition of spoilage has been reviewed (Ogrydziak et al., 1982) and is due to a decrease in CO<sub>2</sub> solubility as the temperature increases. However, the 95% CIs for L. monocytogenes in air and MA overlapped while the CIs for APC diverged, even at the elevated temperature.

We inoculated the samples with high ( $10^2$  CFU/g) and low levels (< $10^1$  CFU/g) levels of L. monocytogenes in order to determine the effect of pathogen level in both the MA and air at 27, 10, and 4°C. When stored at 4°C (Figure 3), by day 20, both the low and high L. monocytogenes concentrations reached the same level, indicating that the initial inoculum level had very little, if any, effect on the final L. monocytogenes count. At 10 and 27°C, low initial levels of L. monocytogenes also increased rapidly in both air and the aerobic MA (data not shown).

The effect of the initial poultry APC counts was determined by inoculating two sets of samples which had either low ( $10^4$  CFU/g) or high ( $10^8$  CFU/g) initial levels of APC organisms. The high APC were achieved by allowing lower count chicken to sit refrigerated for several hours prior to inoculation. Both sets were inoculated with low (< $10^1$  CFU/g) levels of L. monocytogenes. The low APC chicken represents acceptable product, while the high APC chicken,

unacceptable. Low versus high initial poultry microflora levels had virtually no effect on L. monocytogenes growth (Figure 4). For example, in chicken samples with a high background microflora stored in air at 10°C, the CFU for L. monocytogenes increased from  $<10^1$  CFU/g at day 0 to 5.69 at day 4. Under the same storage conditions, chicken samples with a low APC counts had L. monocytogenes CFU/g of  $<10^1$  at day 0 and 5.18 at day 4.

We have suggested (Hintlian and Hotchkiss, 1986) that the ratio of the log of CFU/g for spoilage organisms to pathogenic organisms might be a measure of the relative risk of (not absolute safety or risk) associated with C/MAP or other related technologies when compared to conventional packaging. This ratio was calculated for APC:L. monocytogenes in the aerobic MA and air at three temperatures (Figure 5). Two observations can be made. First, at all temperatures, the ratio of APC to L. monocytogenes in air is greater than in the aerobic MA. This suggests that chicken which contains high numbers of L. monocytogenes is more likely to be spoiled if stored in air than the MA. Second, the ratio in the MA becomes closer to that in air as the temperature increases. This results from the fact that C/MAP is less effective at controlling spoilage as the storage temperature increases.

## CONCLUSIONS

These data illustrate a specific product-MAP-pathogen combination where the growth of spoilage organisms is severely inhibited while

the growth of a pathogen is uninhibited. As we have pointed out (Hintlian and Hotchkiss, 1986) this may be the most hazardous of all situations because the normal cues for spoilage may be absent while the product is pathogenic. It should be pointed out, however, that poultry is rarely, if ever, consumed raw and that L. monocytogenes would be killed by normal cooking procedures. Nonetheless, consumption of uncooked hot dogs or undercooked poultry may be the largest single identifiable risk factor for listeriosis (Schwartz et al., 1988) and there has been a recent confirmed case of listeriosis attributed to L. monocytogenes contamination of a processed poultry product that was apparently properly refrigerated (MMWR, 1989). These epidemiological data, coupled to our experimental results, suggest that raw and processed poultry, especially if packaged under MA, should be carefully monitored for even low levels of L. monocytogenes.

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Table 1. CFU/g of L. monocytogenes and APC from raw chicken packaged in air or 75:25, CO<sub>2</sub>:N<sub>2</sub> (AnMA). Each point represents the mean of four values (duplicate platings of two independent experiments).

Temperature (°C)	Days	<u>L. mono.</u>		<u>APC</u>	
		AnMA	Air	AnMA	Air
27	0.00	2.90	2.96	4.39	4.24
27	0.17	2.93	3.55	4.58	5.94
27	0.33	2.67	5.85	4.45	6.84
27	0.75	2.57	7.82	4.28	9.52
27	1.00	2.45	8.17	4.24	10.40
27	1.25	2.23	8.95	4.10	12.70
10	0	2.77	2.08	4.16	4.30
10	1	2.83	3.48	4.74	4.47
10	2	2.30	4.18	4.85	5.42
10	3	2.17	4.69	3.62	5.94
10	4	2.00	5.23	3.05	6.74
10	5	1.89	6.30	2.47	7.00
4	0	2.91	2.19	4.29	4.18
4	1	2.57	2.21	3.64	4.27
4	2	2.27	2.33	2.88	4.35
4	3	2.27	2.34	2.00	4.53
4	4	1.81	2.66	1.49	4.54
4	5	1.69	2.87	1.40	4.79
4	6	<1.00	3.62	<1.00	4.94

Table 2. CFU/g of L. monocytogenes and APC from raw chicken packaged in air or 72.5:22.5:5, CO<sub>2</sub>:N<sub>2</sub>:O<sub>2</sub> (AeMA). Each point represents the mean of four values (duplicate platings of two independent experiments).

Temperature (°C)	Days	<u>L. mono.</u>		<u>APC</u>	
		AeMA	Air	AeMA	Air
27	0.00	2.48	2.68	4.72	4.69
27	0.17	3.45	3.27	4.74	5.23
27	0.29	4.00	4.00	6.22	6.82
27	0.79	7.00	7.64	9.15	10.50
27	1.08	8.10	9.04	9.35	11.20
27	1.67	9.35	9.72	10.60	13.00
10	0	2.57	2.60	4.00	4.60
10	1	2.74	3.00	4.57	5.00
10	2	3.00	3.06	4.76	5.51
10	3	4.62	4.88	4.79	6.05
10	4	5.04	5.00	5.76	6.79
10	5	5.66	5.79	5.88	8.42
10	6	6.57	6.46	7.57	9.00
10	7	7.00	6.98	7.87	9.80
10	8	7.58	7.63	8.00	10.30
4	0	2.45	2.69	4.42	4.63
4	2	2.51	2.74	4.20	4.28
4	4	2.78	2.80	4.31	4.38
4	6	2.81	3.37	4.37	4.88
4	10	5.78	5.58	4.48	6.52
4	12	6.87	6.75	4.52	6.92
4	14	7.29	7.40	4.68	7.81
4	21	8.40	8.60	5.16	9.27

## Figure Legends

Figure 1. Regression equations (solid lines) and 95% confidence intervals (dashed lines) for the log growth phase of aerobic plate counts on raw chicken in air (solid circles) or modified atmosphere containing 72.5:22.5:5, CO<sub>2</sub>:N<sub>2</sub>:O<sub>2</sub> (open circles) and stored at 4°C.

Figure 2. Regression equations (solid lines) and 95% confidence intervals (dashed lines) for the log growth phase of L. monocytogenes inoculated onto raw chicken at <10<sup>1</sup> CFU/g in air (solid circles) or modified atmosphere containing 72.5:22.5:5, CO<sub>2</sub>:N<sub>2</sub>:O<sub>2</sub> (open circles) and stored at 4°C. The regression lines and 95% CIs are nearly identical in both atmospheres.

Figure 3. Growth curves (4°C) of L. monocytogenes inoculated onto raw chicken at 10<sup>2</sup> (open circles) and <10<sup>1</sup> CFU/g (solid circles).

Figure 4. Growth curves (4°C) of L. monocytogenes inoculated (<10<sup>1</sup> CFU/g) onto raw chicken with low (10<sup>4</sup> CFU/g, solid circles) and high (10<sup>8</sup> CFU/g, open circles) aerobic plate counts.

Figure 5. Ratio of LOG aerobic plate counts to LOG CFU L. monocytogenes in raw chicken at 4, 10, and 27 °C after 21, 8, and 1.67 days of storage, respectively, in air (cross hatched) or in 72.5:22.5:5, CO<sub>2</sub>:N<sub>2</sub>:O<sub>2</sub> (open).









